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(56) Documents cited GB 1562607
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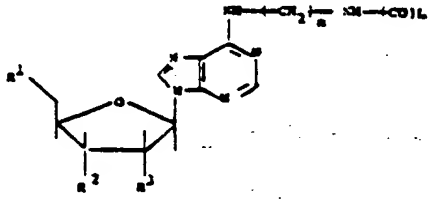
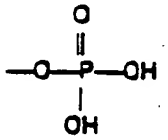
(71) Applicant
Miles Laboratories Inc., 1127 Myrtle Street, Elkhart, Indiana 46514, U.S.A.
(72) Inventors:
Robert Joseph Carrico, Richard Don Johnson
(74) Agent
J. A. Kemp & Co

(54) Intermediates Useful in the Preparation of Flavin Adenine Dinucleotide-labeled Conjugates for use in Specific Binding Assays

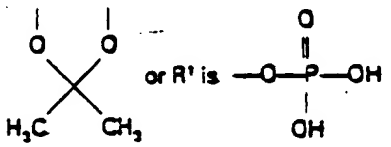
(57) Compounds of the formula:



wherein —(—CO)L is a specifically bindable ligand, or a binding analog thereof, preferably an iodothyronine such as thyroxine, bound through an amide bond; n=2 to 6; R^1 is —OH or

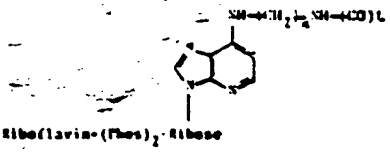


when R^2 and R^3 together form the group



or R^1 is

when R^2 and R^3 are —OH, are useful intermediates in the preparation of labeled conjugates of the formula:



wherein Riboflavin-(Phos)2-Ribose represents the riboflavin-pyrophosphate-ribose residue in flavin adenine dinucleotide (FAD), and n and —(—CO)L are as hereinbefore defined. The FAD-labeled conjugates are useful as labeled conjugates in specific binding assays for determining the ligand or a specific binding partner thereto in ligand media such as serum.

SPECIFICATION

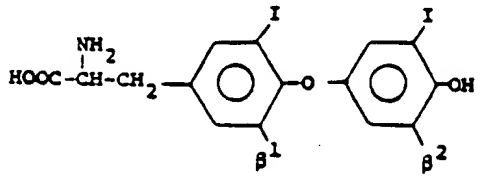
Int rmediates Us ful in The Preparati n of Flavin Adenine Dinucleotide-Labeled C njugates For Use In Specific Binding Assays

Background of the Invention

1. Field of the Invention

This invention relates to intermediates useful in the preparation of labeled conjugates for use in specific binding assays for ligands or their binding partners in a liquid medium, and more particularly for determining an iodothyronine such as thyroxine in serum. This application is a divisional of our Application No. 79,21694. Serial No. 2023609.

The iodothyronines have the following general formula:



wherein β^1 and β^2 are, independently, hydrogen or iodine. The principle iodothyronines of clinical interest are listed in Table 1 below.

Table 1

Iodothyronine	β^1	β^2
3,5,3'5'-tetraiodothyronine (thyroxine; T-4)	iodine	iodine
3,5,3'-triiodothyronine (liothyronine; T-3)	iodine	hydrogen
3,3',5'-triiodothyronine ("reverse" T-3)	hydrogen	iodine
3,3'-diiodothyronine	hydrogen	hydrogen

The quantitative determination of the concentration of the various iodothyronines, particularly th hormones T-3 and T-4, in serum and of the degree of saturation of the iodothyronine binding sites n the carrier protein thyroid binding globulin (TBG) are valuable aids in the diagnosis of thyroid disorders. Likewise, the determination of other components of body fluids including serum is useful in assessing the well-being of an individual. Examples of other substances of clinical interest are evident from the description below.

2. Brief Description of the Prior Art

Specific binding assay methods have undergone a technological evolution from the original competitive binding radioimmunoassay (RIA) in which a radioisotope-labeled antigen is made to compete with antigen from a test sample for binding to specific antibody. In the RIA technique, sampl antigen is quantitated by measuring the proportion of radioactivity which becomes associated with th antibody by binding of the radiolabeled antigen (the bound-species of the labeled antigen) to the radioactivity that remains unassociated from antibody (the free-species) and then comparing that proportion to a standard curve. A comprehensive review of the RIA technique is provided by Sk lly et al, Clin. Chem. 19: 146(1973). While by definition RIA is based on the binding of specific antibody with an antigen or hapten, radiolabeled binding assays have been developed based on other specific binding interactions, such as between hormones and their binding proteins.

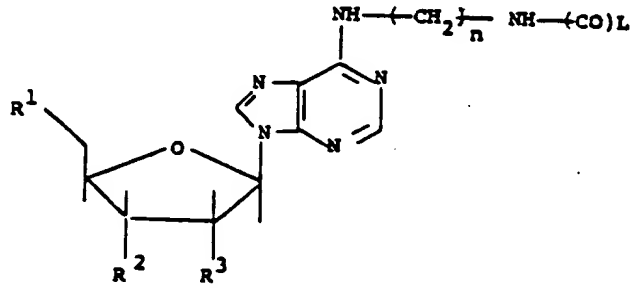
From the radiolabeled binding assays have evolved non-radioisotopic binding arrays employing labeling substances such as enzymes as d scribed in U.S. Patents Nos. 3,654,090 and 3,817,837. Recently further improved nonradioisotopic binding assays hav been developed as described in G rman Offenlegungschriften Nos. 2,618,419 and 2,618,511, based on U.S. Serial Nos. 667,982 and 667,998, filed on March 18, 1976 and assigned to the present assignee, employing particularly uniqu labeling substances, including coenzymes, cyclic reactants, cleavable fluorescent enzyme substrates, and chemiluminescent molecules. Flavin adenine dinucl otide is mentioned as being useful as a coenzyme label since FAD functi ns as a coenzyme in useful monitoring reactions. In U.S. Patent Application Serial No. 917,961, filed June 22, 1978 and assigned to th pres nt assigne

(corresponding to our British Applicati n No. 7921693) Serial No. 2023607 FAD is further described as useful in improved specific binding assays employing a prosthetic group as the label because FAD also functions as a prosthetic group in select biochemical systems.

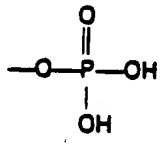
Various methodologies exist for the determination of iodothyronine concentrations in the serum. A significant advance in iodothyronine assays was the development of the competitive protein binding assay by Murphy and Pattee, J. Clin. Endocrinol. Metab. 24:187(1964) in which radiolabeled iodothyronine competes with serum iodothyronine for binding to TBG. The development of specific antiserum for the various iodothyronines permitted radioimmunoassays to be devised in which radiolabeled and serum iodothyronine compete for binding to antibodies rather than to TBG. In both th competitive protein binding assay and the radioimmunoassay for an iodothyronine, the radiolabeled material consists of the native iodothyronine in which one or more of the iodine atoms are replaced by a radioactive iodine isotope, usually ^{125}I. The above-mentioned nonradioisotopic binding assays have offered even more advantageous methods for determining iodothyronines, particularly those methods described in U.S. Patents Nos. 4,043,872 and 4,040,907 and most especially in OLS's 2,618,419 and 2,618,511 and U.S. Serial No. 917,961 mentioned above.

Summary of the Invention

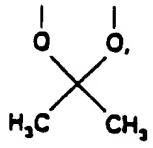
The novel intermediates of the present invention have the formula:



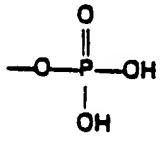
wherein —(—CO)L is a specifically bindable ligand, or a binding analog thereto, and preferably is of formula (5), bound through an amide bond; n=2 through 6; β^1 and β^2 are, independently, hydrogen or iodine; R^1 is —OH or



when R^2 and R^3 together form the group

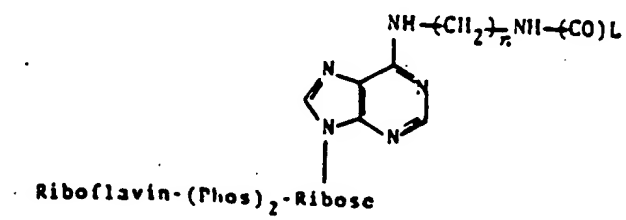


or R^1 is



when R^2 and R^3 are —OH.

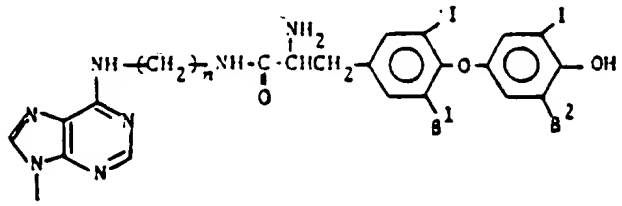
These intermediates, corresponding to formulae (3), (6), and (7) below, are useful in the preparation of novel flavin adenine dinucleotide (FAD)—labeled conjugates for use in binding assays for determining ligands or binding partners thereof, of analytical interest such as the iodothyronin s, and particularly for use in the assay referred to hereinbefore employing a prosthetic group label. The FAD-labeled conjugates have the general formula:



wherein Riboflavin-(Phos)$_2$-Rib se represents the riboflavin-pyrophosphate-ribos residue in FAD; n=2 through 6, and pref rably is 2 or 6; and —(—CO)L is a specifically bindable ligand, or a specific binding analog thereof, and pref rably is an iodothyronine such as thyroxine, bound thr ugh an amide bond.

The specifically bindable ligand or analog thereof in the present labeled conjugates, in terms of its chemical nature, usually is a protein, polypeptide, peptide, carbohydrate, glycoprotein, steroid, or other organic molecule for which a specific binding partner is obtainable. In functional terms, the ligand will usually be an antigen or an antibody thereto; a hapten or an antibody thereto; or a hormone, vitamin, or drug, or a receptor or binding substance therefor. Most commonly, the ligand is an immunologically-active polypeptide or protein of molecular weight between 1,000 and 4,000,000 such as an antigenic polypeptide or protein or an antibody; or is a hapten of molecular weight between 100 and 1,500.

FAD-labeled conjugates wherein the ligand therein is an iodothyronine are particularly useful in specific binding assays to determine the iodothyronine in liquid media such as serum and preferably have the general formula:

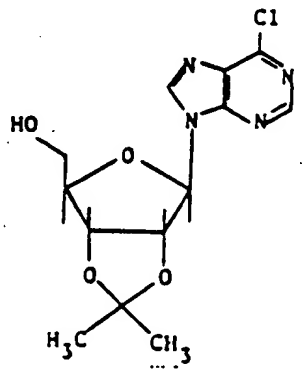


Riboflavin-(Phos)$_2$-Ribose

wherein Riboflavin-(Phos)$_2$-Ribose represents the riboflavin-pyrophosphate-ribose residue in flavin adenine dinucleotide, n=2 through 6, and β^1 and β^2 are, independently, hydrogen or iodine.

The FAD-labeled conjugates are used in binding assays for the ligand or a specific binding partner therefor and are determined, i.e., monitored, for the purposes of the assay by measuring FAD activity, e.g., the coenzyme or prosthetic group activity generated upon combination of such conjugate with an apoenzyme that requires FAD to perform its catalytic function as described in detail in the above-mentioned U.S. Serial No. 917,961.

The present FAD-labeled conjugates can be prepared by a variety of synthetic routes. Exemplary of such available synthetic routes is the following general reaction procedure:



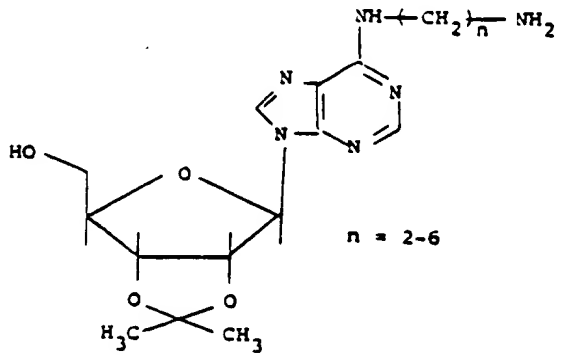
(1)

Reaction of 6-chloro-9-(2',3'-O-isopropylidine-β-D-ribofuranosyl) purine (1) [Hampton *et al, J. Am. Chem. Soc. 83*:150(1961)] with an α,ω-diaminoalkane selected from those listed in Table 2

Table 2

n	*α,ω-diaminoalkane*
2	1,2-diaminoethane
3	1,3-diaminopropane
4	1,4-diaminobutane
5	1,5-diaminopentane
6	1,6-diaminohexane

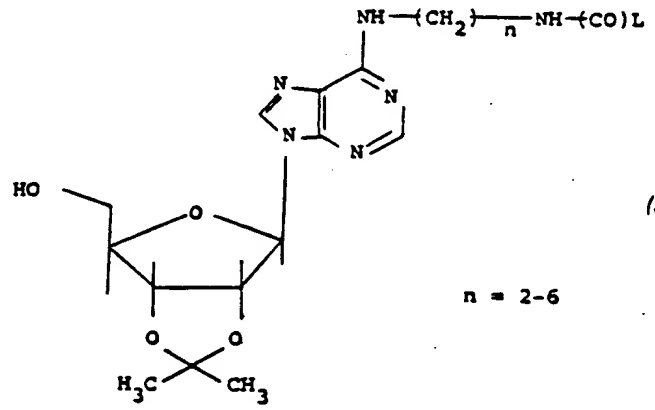
yi lds th intermediate 6-(ω-aminoalkyl)-9-(2',3'-O-isopropylidine-β-D-ribofuranosyl) purine (2).



(2)

n = 2-6

The amino-purine intermediate (2) is then linked by formation of a peptide or amide couple with either the ligand, where such contains a carboxylic acid function, or a binding analog of the ligand (e.g., a derivative of the ligand) which analog contains the desired carboxylic acid function, to form the ligand or analog substituted adenosine intermediate of the present invention of formula (3)



(3)

n = 2-6

wherein —(—CO)L is the ligand or analog thereof bound by an amide bond. Such condensation reactions can be accomplished by reacting the amino-purine intermediate (2) directly with the carboxylic acid-containing ligand or ligand analog using conventional peptide condensation reactions such as the carbodiimide reaction [*Science 144*:1344(1964)], the mixed anhydride reaction [Erlang r *et al, Methods In Immunology and Immunochemistry,* ed. Williams and Chase, Academic Press (New York 1967) p. 149], and the acid azide and active ester reactions [Kopple, *Peptides and Amino Acids,* W. A. Benjamin, Inc. (New York 1966)]. See also for a general review *Clin. Chem. 22*:726(1976).

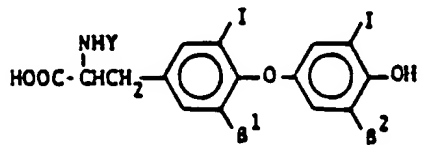
It will be recognized, of course, that other well known methods are available for coupling the ligand or a derivative thereof to the amino-purine intermediate *(2)*. In particular, conventional bifunctional coupling agents can be employed for coupling a ligand, or its derivative, containing a carboxylic acid or amino group to the amino-purine intermediate *(2)*. For example, amine-amine coupling agents such as *bis*-isocyanates, *bis*-imidoesters, and glutaraldehyde *[Immunochem. 6*:53(1969)] can be used to couple a ligand or derivative containing an amino group to the amino-purine intermediate *(2)*. Also, appropriate coupling reactions are well known for inserting a bridg group in coupling an amine (e.g., the amino-purine intermediate) to a carboxylic acid (e.g., the ligand or a derivative thereof). Coupling reactions of this type are thoroughly discussed in the literature, for instance in the above-mentioned Kopple monograph and in Lowe & Dean, *Affinity Chromatography,* John Wiley & Sons (New York 1974).

Such coupling techniques will be considered equivalents to the previously discussed peptide condensation reactions in preparing useful labeled conjugates. The choice of coupling technique will depend on the functionalities available in the ligand or analog thereof for coupling to the amino-purine intermediate *(2)* and n the length f bridging group desired. In all cases, for purposes of this disclosure, th resulting condensati n product will comprise th amino-purine intermediate, which ultimately is c nverted to FAD, bound to the remaining portion f the product, or ultimately to the remaining portion f th FAD-lab led conjugate, through an amine bond. Such remaining porti n f the condensation product, or conjugate, will be considered as a residue of a binding analog of th ligand, unless the ligand itself is directly coupled to th amino-purine intermediate *(2)*. Thus, in this description and in th claims t follow, the abbreviati n —(—CO)L represents the ligand or a binding analog thereof coupled through an amide bond, wh rein such analog can be a derivativ of th ligand coupled by p ptide

condensation or can be th ligand or derivative thereof coupled through a bridging group inserted by coupling of the ligand or d rivative with a bifunctional coupling agent.

It is evident that in coupling the ligand r derivativ thereof to the amino-purine interm diate *(2)* it may be desirable to protect certain reactive groups in such ligand or derivative from participating in side reactions during coupling. Protection of reactive groups may also be desirable to prevent interfering reactions during the synthetic steps described below for completing the preparation of the FAD-labeled conjugate. Depending upon the specific ligand or derivative involved and the coupling technique chosen, the addition of protecting groups at the reactive sites on the ligand or derivative can be accomplished before or after the coupling to the amino-purine intermediate *(2)*. One skilled in the art will have a wide variety of conventional blocking reactions from which to accomplish the desired protection of reactive groups such that the blocking group added can be readily removed in a subsequent synthetic step to yield the original ligand or derivative coupled to FAD.

For instance, where the ligand is an iodothyronine, it is preferably treated to protect the amine group prior to condensation or linkage with the amino-purine intermediate. The amine-protected iodothyronine intermediate has the formula:

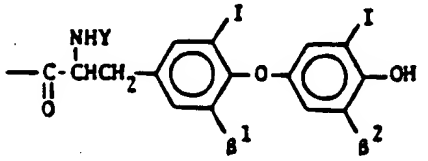


(4)

β^1, β^2 = H or I

wherein Y is an amine-protecting group. It will be recognized that protection of the amine group is a conventional procedure and the amine-protecting group can be selected from a wide variety of groups, including trifluoroacetyl, which is preferred, and the like, such as others of the acyl type (e.g., formyl, benzoyl, phthalyl, *p*-tosyl, aryl- and alkylphosphoryl, phenyl- and benzylsulfonyl, tritylsulfenyl, *o*-nitrophenyl- sulfenyl and *o*-nitrophenoxyacetyl), those of the alkyl type (e.g., trityl, benzyl and alkylidene) and those of the urethane type (e.g., carbobenzoxy, *p*-bromo-, *p*-chloro- and *p*-methoxycarbobenzoxy, tosyloxyalkyloxy-, cyclopentyloxy-, cyclohexyloxy-, t-butyloxy, 1,1-dimethylpropyloxy, 2-(*p*-biphenyl)-2-propyloxy- and benzylthiocarbonyl.

The substituted adenosine intermediates formed by condensation or linkage between the amino-purine intermediate *(2)* and the amine-protected iodothyronine intermediate *(4)* are of the formula *(3)* wherein —(—CO)L is:

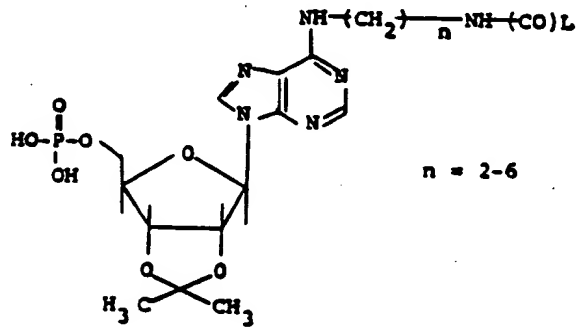


(5)

β^1, β^2 = H or I

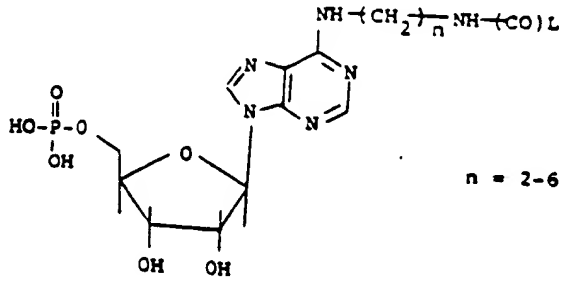
wherein Y is an amine-protecting group as above.

Treatment of intermediate (3) with phosphorous oxychloride produces the phosphorylated ligand or analog substituted adenosine intermediate of the invention of formula (6)



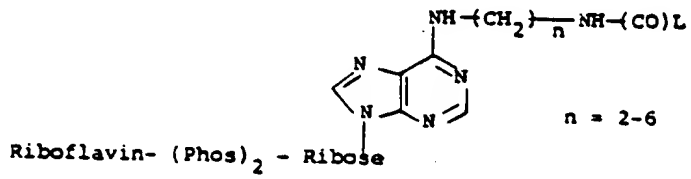
n = 2-6 *(6)*

which upon hydrolysis yields the ligand or analog substituted 5'-adenylic acid intermediate of th invention of formula (7).



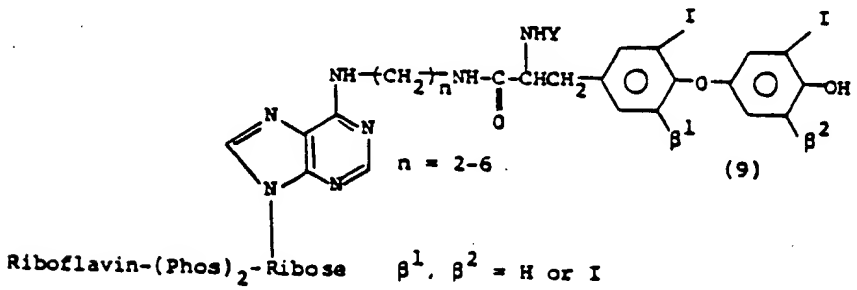
(7)

Condensation of riboflavin-5'-monophosphate with intermediate (7) activated to a phosphorimidazolidate by treatment with N,N'-carbonyldiimidazole yields FAD-labeled conjugates (8).



(8)

In the preferred embodiment wherein the ligand is an iodothyronine, and thus —(—CO)L is represented by formula (5) above, the resulting FAD-iodothyronine conjugates are of the formula:



(9)

wherein Y is an amine-protecting group, or upon conventional treatment for removal of such protecting group, Y is hydrogen.

As stated hereinabove, the ligand which is comprised in the labeled conjugate or whose binding analog is comprised in the labeled conjugate is in most circumstances an immunologically-active polypeptide or protein of molecular weight between 1,000 and 4,000,000, such as an antigenic polypeptide or protein or an antibody, or is a hapten of molecular weight between 100 and 1,500. Various methods for coupling such ligands or analogs thereof to the amino-purine intermediate (2) through an amide bond in the synthesis of the present FAD-labeled conjugate will now be presented.

Polypeptides and Proteins

Representative of specifically bindable protein ligands are antibodies in general, particularly those of the IgG, IgE, IgM and IgA classes, for example hepatitis antibodies; and antigenic proteins such as insulin, chorionic gonadotropin (e.g., HCG), carcinoembryonic antigen (CEA), myoglobin, hemoglobin, follicle stimulating hormone, human growth hormone, thyroid stimulating hormone (TSH), human placental lactogen, thyroxine binding globulin (TBG), instrinsic factor, transcobalamin, enzymes such as alkaline phosphatase and lactic dehydrogenase, and hepatitis-associated antigens such as heptatis B surface antigen (HB_sAg), hepatitis B e antigen (HB_eAg) and hepatitis B core antigen (HB_cAg). Representative of polypeptide ligands are angi tensin I and II, C-peptide, oxytocin, vasopressin, neurophysin, gastrin, secretin, and glucagon.

Since, as peptides, ligands of this general category possess numer us available carboxylic acid and amino groups, coupling to the amino-purin intermediate *(2)* can proceed according to conventional peptide condensation reactions such th carbodiimide reaction, the mixed anhydrid reaction, and so forth as d scribed hereinab ve, or by the us of conventi nal bifunctional reagents capable of coupling carboxylic acid or amin functions to the amino group in the amino-purin intermediates *(2)* as likewise described above. Gen ral references concerning the coupling of proteins to primary amines or carboxylic acids ar mentioned in detail abov .

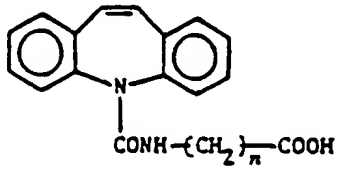
Haptens

Haptens, as a class, offer a wide variety of organic substances which evoke an immunochemical response in a host animal only when injected in the form of an immunogen conjugate comprising the hapten coupled to a carrier molecule, almost always a protein such as albumin. The coupling reactions for forming the immunogen conjugates are well developed in the art and in general comprise the coupling of a carboxylic acid ligand or a carboxylic acid derivative of the ligand to available amino groups on the protein carrier by formation of an amide bond. Such well known coupling reactions are directly analogous to the present formation of labeled conjugates by coupling carboxylic acid ligands or binding analogs to the amino-purine intermediate *(2)*.

Hapten ligands which themselves contain carboxylic acid functions, and which thereby can be coupled directly to the amino-purine intermediate *(2)*, include the iodothyronine hormones such as thyroxine and liothyronine, as well as other materials such as biotin, valproic acid, folic acid and certain prostaglandins. Following are representative synthetic routes for preparing carboxylic acid binding analogs of hapten ligands which themselves do not contain an available carboxylic acid function whereby such analogs can be coupled to the amino-purine intermediate *(2)* by the aforementioned peptide condensation reactions or bifunctional coupling agent reactions (in the structural formulae below, *n* represents an integer, usually 1 through 6, and Me represents methyl).

Carbamazepine

Dibenz[b,f]azepine is treated sequentially with phosgene, an ω-aminoalkanol, and Jones reagent (chromium trioxide in sulfuric acid) according to the method of Singh, U.S. Pat. No. 4,058,511 to yield the following series of carboxylic acids:

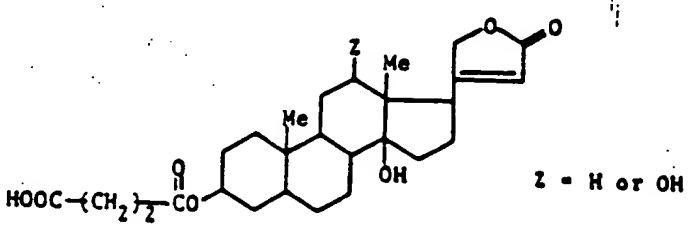


Quinidine

Following the method of Cook *et al, Pharmacologist 17*:219(1975), quinidine is demethylated and treated with 5-bromovalerate followed by acid hydrolysis to yield a suitable carboxylic acid derivative.

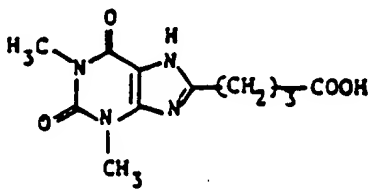
Digoxin and Digitoxin

The aglycone of the cardiac glycoside is treated with succinic anhydride and pyridine according to the method of Oliver *et al, J. Clin. Invest. 47*:1035(1968) to yield the following:



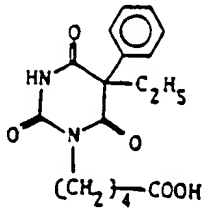
Theophylline

Following the method of Cook *et al, Res. Comm. Chem. Path. Pharm. 13*:497(1976), 4,5-diamino-1,3-dimethylpyrimidine-2,6-dione is heated with glutaric anhydride to yield the following:

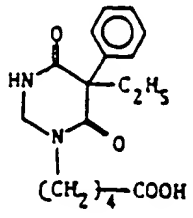


Phenobarbital and Primidone

Sodium phenobarbital is heated with methyl 5-brom valerate and the product hydrolyzed t the corresponding acid derivative of phenobarbital [Cook *et al, Quantitative Analytic Studies in Epilepsy*, ed. K ll way and Pet rson, Raven Press (N w York 1976) pp. 39—58]:

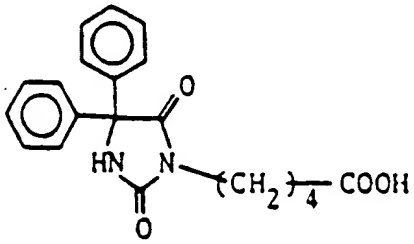


To obtain the acid derivative of primidone following the same Cook *et al* reference method, 2-thiophenobarbital is alkylated, hydrolyzed, and the product treated with Raney nickel to yield:



Diphenylhydantoin

Following the method of Cook *et al, Res. Comm. Chem. Path. Pharm. 5:*767(1973), sodium diphenylhydantoin is reacted with methyl 5-bromovalerate followed by acid hydrolysis to yield the following:

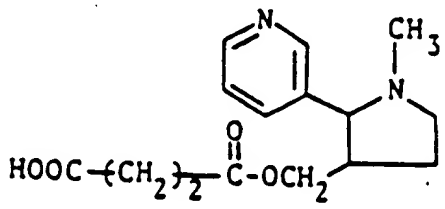


Morphine

Morphine free base is treated with sodium β-chloroacetate according to the method of Spector *et al, Science 168*:1347(1970) to yield a suitable carboxylic acid derivative.

Nicotine

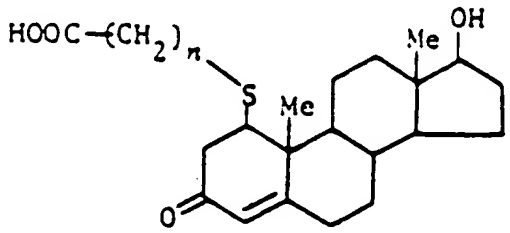
According to the method of Langone *et al, Biochem. 12(24)*:5025(1973), *trans*-hydroxymethylnicotine and succinic anhydride are reacted to yield the following:



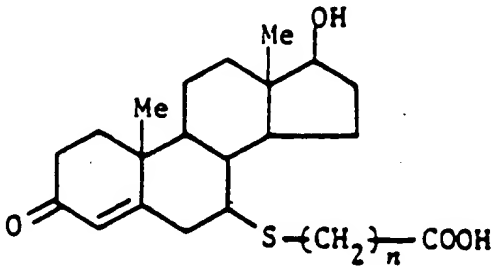
Androgens

Suitable carboxylic acid derivatives of testosterone and androstenedione linked through either the 1- or 7-position on the steroid nucleus are prepared according to the method of Bauminger *et al, J. Steroid Biochem. 5:*739(1974). Following are representativ testosterone derivatives:

1-position

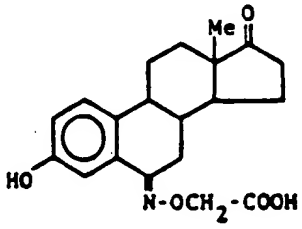


7-position



Estrogens

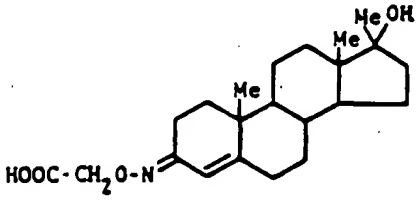
Suitable carboxylic acid derivatives of estrogens, e.g., estrone, estradiol and estriol, are prepared according to the method of Bauminger *et al, supra*, as represented by the following estrone derivativ :



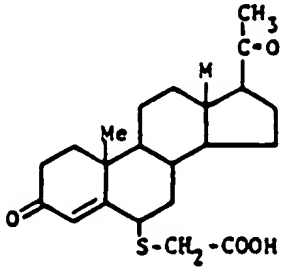
Progesterones

Suitable carboxylic acid derivatives of progesterone and its metabolites linked through any of th 3-, 6- or 7-positions on the steroid nucleus are prepared according to the method of Bauminger *et al, supra*, as represented by the following progesterone derivatives:

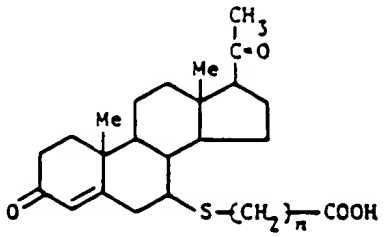
3-position



6-position



7-position



The methods described above are but examples of the many known techniques for forming suitable carboxylic acid derivatives of haptens of analytical interest. The principal derivation techniques are discussed in *Clin. Chem. 22*:726(1976) and include esterification of a primary alcohol with succinic anhydride [Abraham and Grover, *Principles of Competitive Protein-Binding Assays*, ed. Odell and Daughaday, J. B. Lippincott Co. (Philadelphia 1971) pp. 140—157], formation of an oxime from reaction of a ketone group with carboxylmethyl hydroxylamine [*J. Biol. Chem. 234*:1090(1959)], introduction of a carboxyl group into a phenolic residue using chloroacetate [*Science 168*:1347(1970)], and coupling to diazotized *p*-aminobenzoic acid in the manner described in *J. Biol. Chem. 235*:1051(1960).

The general reaction scheme described above is exemplified by the following descriptions of the synthesis of the ethyl (n=2) and hexyl (n=6) analogs of the FAD-labeled conjugates wherein the ligand is the iodothyronine thyroxine [i.e., —(—CO)L is of the formula *(5)* wherein β^1 and β^2 are both iodine]. Also provided are descriptions of assay methods, and results therefrom, employing the exemplified analogs as labeled conjugates in a specific binding assay for thyroxine.

1. Ethyl Analog

1-I. Preparation of the Labeled Conjugate

6-(2-Aminoethyl)amino-9-(2',3'-O-isopropylidine-β-D-ribofluranosyl) purine *(2)*.

13.56 grams (g) [41.5 millimoles (mmol)] of 6-chloro-9-(2',3'-O-isopropylidene-β-D-ribofuranosyl) purine *(1)* [Hampton *et al, J. Am. Chem. Soc. 83*:150(1961)] was added with stirring over a 15 minute period to a cold excess of 1,2-diaminoethane [75 milliliters (ml)]. The resulting solution was allowed to stand at room temperature for 24 hours. The solution was evaporated *in vacuo* and the resulting yellow oil was stirred with 50 ml of cold, saturated sodium bicarbonate. The mixture was evaporated *in vacuo* and the resulting residue was further repeatedly evaporated *in vacuo* first from water (3 times from 50 ml) and then from 2-propanol (4 times from 50 ml) to obtain a yellow glass (15 g). A portion (3 g) of the glass was dissolved in a small volume of water which was then applied to the top of a 25×55 centimeter (cm) Dowex 50W-X2 cation exchange column in the ammonium form (Bio-Rad Laboratories, Richmond, California USA).

The column was eluted with a linear gradient generated with 2 liters (L) each of water and 0.5 molar (M) ammonium bicarbonate. The elution was completed using a linear gradient generated with 2 L each of 0.5 M and 1 M ammonium bicarbonate. The effluent from the column was collected in 19 ml fractions and monitored by elution on silica gel thin layer chromatography (TLC) plates (E. Merck, Darmstadt, West Germany) with a 9:1 (V:v) mixture of ethanol and ammonium hydroxide. The developed TLC plates were examined under ultraviolet light, then sprayed with ninhydrin reagent [Randerath, *Thin Layer Chromatography*, Academic Press (1966)]. Fractions numbered 250 through 350 from the column chromatography were combined and evaporated *in vacuo* leaving the desired purine *(2)* as a pale yellow amorphous glass (1.5 g).

Analysis:

Calculated for $C_{15}H_{22}N_6O_4$:
C, 51.42; H, 6.33; N, 23.99

Found:
C, 50.92; H, 6.54; N, 23.01

NMR (60 MHz, $CDCl_3$): δ 1.37 (s, 3H, isopropylidene), 1.63 (s, 3H, isopropylidene), 5.92 (d, 1H, 1'-ribose), 7.90 (s, 1H, purine), 8.26 (s, 1H, purine) Optical Rotation $[\alpha]_D^{20}$=—74.85° (c 1.0, CH_3OH)

Th remaining crude product (12 g) was purified by chromatography on Dowex 50W-X2 as described abov . Th overall yield was 8 g (55%).

α-(N-Trifluoroacetyl)amino-β-[3,5-dii do-4-(3',5'-diiod -4'-hydroxyphenoxy)phenyl] propanoic acid *(4)*.

This compound was prepared by the m thod f Blank, *J. Pharm. Sci. 53*:1333(1964). To a cooled (0°C), stirred suspensi n of 5 g (6.4 mm l) of L-thyroxine (Sigma Chemical Co., St. Louis, Missouri

USA) in 60 ml of dry ethyl acetate was added 11.5 ml of trifluoroacetic acid and 1.9 ml of trifluoroacetic anhydride. After 30 minutes the resulting clear soluti n was wash d three times with 30 ml of water, once with 30 ml of 5% sodium bicarbonate, and twice with 50 ml of saturated sodium chloride. The combined aqueous washings were extracted twice with 20 ml of ethyl acetate. The ethyl acetate layers were combined and washed with 30 ml of water, then dried over magnesium sulfate. The dried ethyl acetate solution was evaporated *in vacuo* leaving a white solid. Recrystallization fr m a mixture of ethyl ether and petroleum ether gave a pinkish-white solid (3.95 g, 70.5% yield) having a melting point (m.p.) of 228—230°C with decomposition.

Analysis:
Calculated for $C_{17}H_{10}F_3I_4NO_5$:
C, 23.39; H, 1.15; N, 1.60
Found:
C, 23.00; H, 1.05; N, 1.65
NMR [60 MHz, $DCON(CD_3)_2$] δ7.28 (s, 2H, aromatic), 8.03 (s, 2H, aromatic), 9.7 (m, 1H, amido)
IR (KCl): 1700 (>C=O) Optical Rotation $[\alpha]_D^{25}$=—14.97° (c 1.0 dimethylsulfoxide)
A second recrystallization produced a second precipitate (0.95 g) m.p. 224—228°C with decomposition. The overall yield was 87.5%.

N-[2-[N-(Trifluoroacetyl)-3,3',5,5'-tetraiodothyronyl]aminoethyl]-2',3'-O-isopropylidene adenosine *(3)*.

A solution of 8.72 g (10.0 mmol) of α-(N-trifluoroacetyl)-amino-β-[3,5-diiodo-4-(3',5'-diiodo-4'-hydroxyphenoxy)phenyl]propanoic acid *(4)* and 3.86 g (11.0 mmol) of 6-(2-aminoethyl) amino-9-(2',3'-O-isopropylidene-β-D-ribofuranosyl) purine *(2)* in 50 ml of dry dimethylacetamide was prepared under a dry argon atmosphere at —20°C. To this cold stirred solution was added a solution of 3.04 g (11.0 mmol) of diphenylphosphoryl azide (Aldrich Chemical Co., Milwaukee, Wisconsin USA) in 10 ml of dry dimethylacetamide followed by the addition of 1.6 ml (11.0 mmol) of dry triethylamine. The solution was left at room temperature for 22 hours. The solution was then added dropwise to 300 ml of cold (0°C) water with stirring. The resulting white precipitate was collected by filtration and dried *in vacuo* (56°C) to give 13.0 g of a light cream colored solid. The solid was dissolved in 500 ml of acetone and the solution was concentrated by boiling. The white solid which precipitated from the boiling acetone solution was collected by filtration while hot. Continued boiling of the filtrate produced two additional precipitates. The three precipitates were combined to give 8 g (66.6% yield) of a white solid, m.p. 198—200°C (decomposed).

Analysis:
Calculated for $C_{32}H_{30}F_3I_4N_7O_8$:
C, 31.89; H, 2.51; N, 8.14
Found:
C, 31.95; H, 2.60; N, 7.86
NMR [220 MHz, $(CD_3)_2SO$] δ 1.32 (s, 3H, isopropylidene), 1.55 (s, 3H, isopropylidene), 6.14 (d, 1H, 1'-ribose), 7.02 (s, 2H, thyroxine), 7.82 (s, 2H, thyroxine), 8.25 (s, 1H, purine), 8.36 (s, 1H, purine), 8.41 (t, 1H, J=6, amido), 9.64 (d, 1H, J=8, trifluoroacetamido)
Optical Rotation $[\alpha]_D^{25}$=—11.82° (c 1.0, pyridine)

N-[2-[N-(Trifluoroacetyl)-3,3',5,5'-tetraiodothyronyl]aminoethyl]-2',3'-O-isopropylidene-5'-adenylic acid monotriethylamine salt monohydrate *(6)*.

A solution of 1.2 g (1.0 mmol) of N-[2-[N-(trifluoroacetyl)-3,3',5,5'-tetraiodothyronyl]aminoethyl]-2',3'-O-isopropylidene adenosine *(3)* in 10 ml of dry triethylphosphate was prepared under a dry argon atmosphere at 0°C. To the cold, stirred solution was added 0.45 ml (5 mmol) of phosphorous oxychloride. The resulting solution was kept for 24 hours at 0°C, then added dropwise with stirring to 1 L of ice water. The resulting precipitate was collected by filtration and dried *in vacuo* to give 1.23 g of a white solid. The solid was dissolved in acetone and 0.32 ml (2.2 mmol) of triethylamine was added. A precipitate formed. The mixture was evaporated *in vacuo* and the resulting residue lixiviated with dry acetone, then recrystallized from a mixture of dry methyl alcohol and dry ethyl ether to give 390 mg (27.8% yield) of a white solid, m.p. 173—183°C (decomposed).

Analysis:
Calculated for $C_{38}H_{48}F_3I_4N_8O_{12}P$:
C, 32.50; H, 3.45; N, 7.98
Found:
C, 32.24; H, 3.08; N, 7.58
NMR [60 MHz, $(CD_3)_2SO$] δ 1.53 (s, 3H, isopropylidene), 6.2 (d, 1H, 1'H-ribose), 7.1 (s, 2H, thyroxine aromatic), 7.87 (s, 2H, thyroxine aromatic), 8.27 (s, 1H, purine), 8.52 (s, 1H, purine)
Optical Rotation $[\alpha]_D^{25}$=—17.50° (c 1.0, CH_3OH)

N-[2-[N-(Trifluoroacetyl)-3,3',5,5'-tetraiodothyronyl]aminoethyl]-5'-adenylic acid *(7)*.

200 milligrams (mg) (0.14 mmol) of N-[2-[N-(trifluoroacetyl)-3,3'-5,5'-tetraiodothyronyl]aminoethyl]-2',3'-O-isopropylidene-5'-adenylic acid monotriethylamine salt monohydrate *(6)* was suspended in 1 ml of water (0°C) and trifluoroacetic acid (9 ml) was added dropwise with stirring. After 30 minutes a clear solution was obtained. The solution was kept cold (0°C) for an additional 15 hours, then evaporated *in vacuo* (30°C). The resulting residue was evaporated four times *in vacuo* (25°C) from 20 ml volumes of anhydrous ethyl alcohol and then dried *in vacuo* (25°C) leaving a white solid.

The solid was stirred for 30 minutes with 10 ml of cold methyl alcohol, then collected by filtration and dried *in vacuo* (25°C) to give a white solid (135 mg, 76% yield) which slowly melted with decomposition above 188°C.

Analysis:

Calculated for $C_{29}H_{27}F_3I_4N_7O_{11}P$:
C, 27.97; H, 2.19; N, 7.87

Found:
C, 28.11; H, 2.31; N, 7.65

NMR [220 MHz, $(CD_3)_2SO$] δ 5.95 (d, 1H, 1'-ribose), 7.04 (s, 2H, thyroxine aromatic), 7.84 (s, 2H, thyroxine aromatic), 8.25 (s, 1H, purine), 8.36 (s, 1H, purine), 8.43 (m, 1H, amido), 9.66 (d, 1H, trifluoroacetamido)

Optical Rotation $[\alpha]_D^{25} = -2.72°$ (c 1.0, pyridine)

Flavin adenine dinucleotide-thyroxine conjugate *(8)*.

498 mg (0.4 mmol) of N-[2-[N-(trifluoroacetyl)-3,3',5,5'-tetraiodothyronyl]aminoethyl]-5'-adenylic acid *(7)* was dissolved in 10 ml of dry dimethylformamide and tri-n-butylamine [96 microliters (μl), 0.4 mmol] was added followed by the addition of 1,1'-carbonyldiimidazole (320 mg, 2.0 mmol). After stirring for 18 hours at room temperature in the absence of moisture, water (280 μl) was added and then the solvent evaporated *in vacuo*.

The resulting oil was dried by repeated *in vacuo* evaporation from dry dimethylformamide (4 times from 10 ml). The resulting phosphorimidazolidate was redissolved in 10 ml of dry dimethylformamide and added dropwise to a 0.4 mmol solution of the tri-n-octylamine salt of riboflavin-5'-monophosphate in 10 ml of dry dimethylformamide. The salt was prepared by adding a solution of the ammonium salt of riboflavin-5'-monophosphate (192 mg, 0.4 mmol) in 10 ml of water to a stirred solution of tri-n-octylamine (176 μl, 0.4 mmol) in 100 ml of acetone. After 30 minutes, the resulting mixture was evaporated *in vacuo*. The residue was dried by repeated evaporation *in vacuo* from dry dimethylformamide leaving the salt as an orange solid.

The above solution containing the phosphorimidazolidate of *(7)* and the riboflavin-5'-monophosphate salt was divided into two equal aliquots after 24 hours and one aliquot was evaporated *in vacuo*. The resulting residue was chromatographed on a column (2.5x78 cm) prepared from 100 g of Sephadex LH-20 (Pharmacia Fine Chemicals, Uppsala, Sweden) which had been preswollen (18 hours) in a 19:1 (v:v) mixture of dimethyl-formamide and triethylammonium bicarbonate (1 M, pH 7.5). The column was eluted with the above 19:1 (v:v) mixture and 10 ml fractions were collected. The effluent from the column was monitored by elution on silica gel 60 silanised RP-2 TLC places (E. Merck, Darmstadt, West Germany).

The TLC plates were developed using a 40:40:25:1:1 (v:v) mixture of acetone, chloroform, methyl alcohol, water, and triethylamine. Fractions numbered 11 through 17 from the above-mentioned column chromatography were combined and evaporated *in vacuo*. The residue was chromatographed on a column (2.5x75 cm) prepared from 125 g of Sephadex LH-20 which had been preswollen (18 hours) in 0.3 M ammonium bicarbonate. The column was eluted with 0.3 M ammonium bicarbonate collecting 10 ml fractions. The effluent was monitored by absorption of ultraviolet light at 254 nanometers (nm). The volume of the fractions was increased to 20 ml beginning with fraction number 150. The salt concentration of the eluent was decreased in a stepwise fashion as follows: 0.15 M ammonium bicarbonate at fraction number 295, 0.075 M ammonium bicarbonate at fraction number 376, and water at fraction number 430. A total of 480 fractions was collected. Fractions numbered 200 through 235 were combined and evaporated *in vacuo* leaving the labeled conjugate *(8)* as a yellow-orange residue. An alkaline, aqueous solution of this residue exhibited ultraviolet absorption maxima at the following wavelengths: 266 nm, 350 nm, 373 nm, and 450 nm. The yield, estimated from the absorption at 450 was about 5%.

A phosphodiesterase preparation (Worthington Biochemical Corp., Freehold, New Jersey USA) isolated from snake venom *(Crotalus Adamanteus)* hydrolyzed and the above product to riboflavin-5'-monophosphate and the thyroxine substituted 5'-adenylic acid *(7)* wherein the trifluoroacetyl blocking group had been removed.

1-II. Binding Assay for Thyr xine

The above-prepared labeled conjugate was us d in a prosthetic group-labeled specific binding assay as follows (furth r details regarding such an assay method may be f und in the U.S. Patent Application—Serial No. 917,961-referred to hereinbefore):

A. Preparation of Apoglucose Oxidase

Purified glucose oxidase with low catalase activity obtained from the Research Products Division of Miles Laboratories, Inc., Elkhart, Indiana USA was twice dialyzed for 12 hours each against 0.5% (w:v) mannitol (30 volumes each). Aliquots of the dialyzate containing 100 mg of glucose oxidase each were lyophilized and stored at −20°C.

Bovine serum albumin (200 mg) was dissolved in 12 ml of water adjusted to pH 1.6 with concentrated sulfuric acid, mixed with 150 mg charcoal (RIA grade from Schwarz-Mann, Orangeburg, New York USA), and cooled to 0°C. Lyophilized glucose oxidase (100 mg) was redissolved in 3.1 ml of water and 3 ml was added to the stirred albumin-charcoal suspension with continued stirring for thre minutes. The suspension was then filtered through a 0.8 micron, 25 millimeters (mm) diameter Millipore filter (millipore Corp., Bedford, Massachusetts USA) mounted in a Sweenex filter apparatus (Millipore Corp.) on a 50 ml disposable plastic syringe. The filtrate was quickly neutralized to pH 7.0 by addition of 2 ml of 0.4 M phosphate buffer (pH 7.6) and thereafter 5 N sodium hydroxide. Dry charcoal (150 mg) was then added and stirred for one hour at 0°C. The resulting suspension was filtered first through a 0.8 micron Millipore filter and then through a 0.22 micron Millipore filter. To the filtrate was added glycerol to 25% (v:v) and the stabilized apoglucose oxidase preparation was stored at 4°C.

B. Assay Reagents

1. Labeled conjugate—The ethyl analog labeled conjugate prepared as in section *1—I* abov was diluted in 0.1 M phosphate buffer (pH 7) to a concentration of 1 micromolar (μM).

2. Apoenzyme—Apoglucose oxidase was diluted with 0.1 M phosphate buffer (pH 7) to a concentration of 0.6 μM FAD binding sites. The FAD binding site concentration of the apoenzym preparation was determined experimentally by measuring the minimum amount of FAD required to give maximum glucose oxidase activity when incubated with the apoenzyme.

3. Insolubilized Antibody—A washed, moist cake of Sepharose 4B gel (Pharmacia Fine Chemicals, Uppsala, Sweden) activated by cyanogen bromide according to the method of March *et al, Anal. Biochem. 60*:119(1974) was added to a solution of 85 mg of antibody, (Isolated from antiserum against a thyroxine-bovine serum albumin conjugate) in 20 ml of 0.1 M phosphate buffer (pH 7.0) and agitated slowly for 36 hours at 4°C. Upon completion of the coupling reaction, 1 ml of 1 M alanine was added and shaking continued for four more hours to block unreacted sites. The resulting Sepharose-bound antibody was washed on a scintered funnel with 400 ml each of 50 mM sodium acetate—500 millimolar (mM) sodium chloride (pH 5) and 50 mM phosphate buffer—500 mM sodium chloride (pH 7), and 800 ml of 100 mM phosphate buffer (pH 7). The moist filter cake was then suspended in 100 mM phosphate buffer (pH 7) containing 0.01% sodium azide to give 22 ml of an about 50% suspension.

4. Standard—A 1.15 mM stock solution of thyroxine in 5 mM sodium hydroxide was diluted to 2 μM in 0.1 M phosphate buffer (pH 7).

5. Monitoring reagent—A glucose oxidase assay reagent was prepared to contain the following mixture per 130 μl: 25 μl of 1.2 mg/ml peroxidase (Sigma Chemical Co., St. Louis, Missouri USA) in 0.1 M phosphate buffer (pH 7), 5 μl of 10 mM 4-aminoantipyrine in water, 20 μl of 25 mM 3,5-dichloro-2-hydroxybenzene sulfonate in 0.1 M phosphate buffer (pH 7), 30 μl of 16.5% bovine serum albumin in 0.1 M phosphate buffer (pH 7), and 50 μl of 1 M glucose in aqueous saturated benzoic acid solution.

C. Assay Procedure

Binding reaction mixtures were prepared by mixing 150 μl of the insolubilized antibody suspension, 80 μl of the labeled conjugate solution, various amounts of the standard thyroxine solution to give varying concentrations of thyroxine in the reaction mixtures, and a sufficient volume of 0.1 M phosphate buffer (pH 7) to male a total volume of 500 μl. The reaction mixtures were incubated with shaking for two hours at 25°C. Each reaction mixture was then vacuum filtered through a glass wool plugged, dry pasteur pipette previously treated sequentially with p riodate and ethylene glycol solutions to eliminate possible FAD contaminati n. To a 300 μl aliquot of each filtrate was added 130 μl of the monitoring reagent and 50 μl of the apoenzym solution. After one hour, th absorbance f each reaction mixture was measured at 520 nm.

D. Results

Following is Table 3 showing the results of th assay procedure in measuring thyroxine. Th absorbanc results are expressed as the average of duplicate runs corr cted for residual enzyme

activity in th ap enzym solution (abs rbance of 0.522) and f r endogenous FAD in the antibody suspensi n (abs rbanc of 0.142).

Tabl 3

Volume of Thyroxine Standard Added (μl)	*Absorbance (520 nm)*
0	0.223
25	0.221
75	0.281
250	0.286

The results demonstrate that the present labeled conjugates are useful in a specific binding assay method for determining a ligand in a liquid medium.

Hexyl Analog

2—I. Preparation of the Labeled Conjugate

6-(6-Aminohexyl)amino-9-(2',3'-O-isopropylidene-β-D-ribofuranosyl) purine *(2)*.

16.0 g (50 mmol) of 6-chloro-9-(2',3'-O-isopropylidene-β-D-ribofuranosyl) purine *(1)* [Hampton *et al, J. Am. Chem. Soc. 83*:1501(1961)] was added with stirring to a molten (70°C) sample of freshly distilled 1,6-diaminohexane (58 g, 500 mmol). The resulting mixture was stirred under argon at 40°C for 18 hours. The excess diamine was removed by distillation under reduced pressure (60°C, 0.01 mm Hg). The resulting pale yellow residue was adsorbed onto 150 g of silica gel 60 (E. Merck, Darmstadt, West Germany) and used to top a chromatographic 9:1 (v:v) mixture of absolute ethyl alcohol and triethylammonium bicarbonate (pH 7.5, 1 M). The column was eluted with the above 9:1 (v:v) solvent mixture and 900 20 ml fractions were collected. The fractions were examined by thin layer chromatography (TLC) on silica gel 60 eluting with a 7:3 (v:v) mixture of absolute ethyl alcohol and triethylammonium bicarbonate (pH 7.5, 1 M). Fractions numbered 391 through 900 from the column chromatography were combined and evaporated *in vacuo* leaving 15.0 g of a glassy residue (74% yield). A 1 g sample of the glass was dissolved in a small volume of methyl alcohol and applied to th top of a column prepared from 80 g of Sephadex LH-20 (Pharmacia Fine Chemicals, Uppsala, Sweden) preswollen in methyl alcohol. The column was eluted with methyl alcohol. A total of ninety 8 ml fractions were collected. The fractions were examined by TLC on silica gel 60 eluting with a 7:3 (v:v) mixture of absolute ethyl alcohol and triethylammonium bicarbonate (pH 7.5, 1 M). Fractions numbered 19 through 27 from the column chromatography were combined and evaporated *in vacuo* leaving 910 mg (91% recovery) of a white glass.

Analysis:

Calculated for $C_{19}H_{30}N_6O_4$:
C, 56.14; H, 7.44; N, 20.68
Found:
C, 53.91; H, 7.33; N, 19.18
NMR (60 MHz, $CDCl_3$): δ 1.40 (s, 3H, isopropylidene), 1.63 (s, 3H, isopropylidene) 5.98 (d, 1H, 1'-ribose), 7.92 (s, 1H, purine), 8.36 (s, 1H, purine)
Optical Rotation $[\alpha]_D^{25}$=−50.11° (c 1.0, methyl alcohol)

N-[6-[N-(Trifluoroacetyl)-3,3',5,5'-tetraiodothyronyl]aminohexyl]-2',3'-O-isopropylidene adenosine *(3)*.

A solution of 4.36 g (5.0 mmol) of α-(N-trifluoroacetyl)amino-β-[3,5-diiodo-4-(3',5'-diiodo-4'-hydroxyphenoxy)-phenyl]propanoic acid *(4)*, prepared as described in section *1—I* above, and 2.24 g (5.5 mmol) of 6-(6-aminohexyl)amino-9-(2',3'-O-isopropylidene-β-D-ribofuranosyl) purine *(2)* in 100 ml of dry dimethylformamide was prepared und r a dry argon atmosphere at −20°C. To this cold stirred solution was added a solution of 1.52 g (5.5 mmol) of diphenylphosphoryl azide (Aldrich Chemical Co., Milwaukee, Wisconsin USA) in 50 ml of dry dim thylformamide followed by the addition of 0.8 ml (5.5 mmol) of dry triethylamin . Th solution was l ft at room temperature for 22 hours. The solution was then added dropwise to 600 ml of cold (0°C) water with stirring. The resulting white precipitate was collected by filtration and dried *in vacuo* (60°C) to giv 4.90 g (78% yield) f white solid. A sampl of this solid was recrystallized from a mixtur of acetone and water giving a white solid, m.p. 205—207°C (decomposed).

Analysis:
Calculated for $C_{36}H_{38}F_3I_4N_7O_9$:
C, 34.8; H, 3.04; N, 7.77
Found:
C, 34.22; H, 2.99; N, 7.41
Mass Spectrum (20 ma) m/e: 1262 [MH^+], 1164 [M^+ minus $COCF_3$]
Optical Rotation $[\alpha]_D^{25}$=−21.89° (c 1.0, pyridine)

N-[6-[N-(Trifluoroacetyl)-3,3',5,5'-tetraiodothyronyl]aminohexyl]-2',3'-O-isopropylidene-5'-adenylic acid monotriethylamine salt monohydrate *(6)*.

A solution of 1.89 g (1.5 mmol) of N-[6-N-(trifuloroacetyl)-3,3',5,5'-tetraiodothyronyl]aminohexyl]-2',3'-O-isopropylidene adenosine *(3)* in 15 ml of dry triethylphosphate was prepared under a dry argon atmosphere at −10°C. To the cold stirred solution was added 0.68 ml (7.5 mmol) of phosphorous oxychloride. The resulting solution was kept for 18 hours at −15°C then added dropwise with stirring to 1.5 L of ice water. The resulting precipitate was collected by filtrati n and dried *in vacuo* to give 1.91 g (87% yield) of a white solid. The solid was dissolved in 10 ml methyl alcohol and 0.38 ml (2.6 mmol) of triethylamine was added. This solution was evaporated *in vacuo* and the resulting residue was recrystallized from a mixture of methyl alcohol and ethyl ether to give 720 mg (33% yield) of a white solid, m.p. 151—154°C (decomposed).

Analysis:
Calculated for $C_{42}H_{56}F_3I_4N_8O_{12}P$:
C, 34.54; H, 3.86; N, 7.67
Found:
C, 35.24; H, 3.88; N, 7.75
Mass Spectrum (20 ma) m/e: 1342 [MH^+], 1244 [M^+ minus $COCF_3$]
Optical Rotation $[\alpha]_D^{25}$=−17.20° (c 1.0, CH_3OH)

N-[6-[N-(Trifluoroacetyl)-3,3',5,5'-tetraiodothyronyl]aminohexyl]-5'-adenylic acid *(7)*.

600 mg (0.41 mmol) of N-[6-[N-(trifluoroacetyl)-3,3',5,5'-tetraiodothyronyl]aminohexyl]-2',3'-O-isopropylidene-5'-adenylic acid monotriethylamine salt monohydrate *(6)* was suspended in 0.6 ml of water (0°C) and trifluoroacetic acid (6 ml) was added dropwise with stirring. After 50 minutes a clear solution was obtained. The solution was kept cold (0°C) for an additional 15 hours then evaporated *in vacuo* (30°C). The resulting residue was evaporated *in vacuo* five times from 20 ml volumes of anhydrous ethyl alcohol then triturated with 30 ml water and washed with a small volume of methyl alcohol. The resulting white solid (430 mg) was recrystallized from methyl alcohol to give 290 mg (54.6% yield) of white solid, m.p. 180—183°C (decomposed).

Analysis: Calculated for $C_{33}H_{39}F_3I_4N_7O_{11}P$:
C, 30.46; H, 2.71; N, 7.54
Found:
C, 30.77; H, 2.55; N, 7.29
Mass Spectrum (20 ma) m/e: 1302 [MH^+], 1204 [M^+ minus $COCF_3$]

Flavin Adenine Dinucleotide—Thyroxine Conjugate *(8)*.

130.13 mg (0.1 mmol) of N-[6-[N-(trifluoroacetyl)-3,3',5,5'-tetraiodothyronyl]aminoh xyl]-5'-adenylic acid *(7)* was placed in an argon atmosphere. To this sample was added a solution of 14 μl (0.1 mmol) of triethylamine in 1 ml of dry dimethylformamide followed by the addition of a solution of 16.2 mg (0.1 mmol) of 1,1'-carbonyldiimidazole in 1 ml of dry dimethylformamide. After 24 hours, a second equivalent of 1,1'-carbonyldiimidazole (16.2 mg) in 1 ml of dry dimethylformamide was added. The above reaction was allowed to proceed a total of 48 hours at room temperature excluding moisture. A sample of 47.3 mg (0.1 mmol) of the ammonium salt of riboflavin-5'-monophosphate was converted to the corresponding tri-n-octylamine salt as described in section *1—I* above. This salt was dissolved in 3 ml of dry dimethylformamide and added to the above solution containing the phosphorimidazolidate of the adenylic acid intermediate *(7)*.

Th resulting solution was allowed to stand in the dark at room temperature excluding moisture for 24 hours. The solvent was evaporated *in vacuo* and th resulting residue was chromatographed on a column (2.5×78 cm) prepared fr m 100 g f Sephadex LH-20 (Pharmacia Fine Chemicals, Uppsala, Sweden) which had b en preswollen (18 h urs) in a 19:1 (v:v) mixture of dimethylformamide and triethylammonium bicarbonate (1 M, pH 7.5). The column was eluted with the above 19:1 (v:v) mixture and 5 ml fractions were collected. Th efflu nt from th column was monitored by elution on silica gel 60 silanised RP-2 TLC plates (E. M rck, Darmstadt, West Germany). The TLC plates wer developed using a 40:40:25:1:1 (v:v) mixture of acet ne, chloroform, m thyl alcoh l, water, and triethylamine.

Fractions numbered 24 through 38 from the column chromatography were combined and

evaporated *in vacuo*. The residue was chromatographed on a column (2.5x85 cm) prepared from 125 g of Sephadex LH-20 which had been preswoll n (18 hours) in 0.1 M ammonium bicarbonate. Th column was eluted with a linear gradient generated from 2L of 0.1 M ammonium bicarb nat and 2 L of water and 23 ml fractions collected. The effluent was monitored by ultraviol t absorpti n (254 nm). Fractions numbered 170 through 182 were combined and evaporated *in vacuo*. The residue was chromatographed on a column (2.5x55 cm) prepared from 80 g of Sephadex LH-20 which had been preswollen in 0.05 M ammonium bicarbonate. The column was eluted with a linear gradient generated from 2 L of 0.05 M ammonium bicarbonate and 2 L of 0.02 M ammonium bicarbonate. The effluent was monitored by ultraviolet absorption (254 nm). Elution was continued with 2 L of 0.2 M ammonium bicarbonate, collecting 23 ml fractions. A total of 257 fractions was collected. Fractions numbered 70 through 110 were combined and evaporated *in vacuo* leaving the labeled conjugate *(8)* as a yellow-orange residue. An alkaline, aqueous solution of this residue exhibited ultraviolet absorption maxima at the following wavelengths: 270 nm, 345 nm, and 450 nm. The yield, estimated from the absorption at 450 nm, was about 5%.

A phosphodiesterase preparation (Worthington Biochemical Corp., Freehold, New Jersey USA) isolated from snake venom *(Crotalus Adamanteus)* hydrolyzed the above product to riboflavin-5'-monophosphate and the thyroxine substituted 5'-adenylic acid *(7)* wherein the trifluoroacetyl blocking group had been removed.

2.—II. Binding Assay for Thyroxine

The above-prepared labeled conjugate was used in a prosthetic-group labeled specific binding assay as follows (further details regarding such an assay method may be found in the U.S. Patent Application—Serial No. 917,961—referred to hereinbefore):

A. Preparation of Apoglucose Oxidase

The apoenzyme used was prepared by the method described in section *1—II*, part A above.

B. Assay Reagents

1. Labeled conjugate—The hexyl analog labeled conjugate prepared as in section *2—I* above was diluted in 0.1 M phosphate buffer (pH 7) to a concentration of 100 nM.

2. Apoenzyme—This reagent was the same as that described in section *1—II*, part B-2 above.

3. Insolubilized antibody—This reagent was the same as that described in section *1—II*, part B-3 above.

4. Standard—A 1.15 mM stock solution of thyroxine in 5 mM sodium hydroxide was diluted to 1 μM in 0.1 M phosphate buffer (pH 7).

5. Monitoring reagent—A glucose oxidase reagent was prepared to contain the following mixture per 117 μl: 25 μl of 1.2 mg/ml peroxidase (Sigma Chemical Co., St. Louis, Missouri USA) in 0.1 M phosphate buffer (pH 7), 5 μl of 10 mM 4-aminoantipyrine in water, 20 μl of 25 mM 3,5-dichloro-2-hydroxybenzene sulfonate in 0.1 M phosphate buffer (pH 7), 17 μl of 30% bovine serum albumin in 0.1 M phosphate buffer (pH 7), and 50 μl of 1 M glucose in aqueous saturated benzoic acid solution.

C. Assay Procedure

Binding reaction mixtures were prepared by mixing 30 μl of the insolubilized antibody suspension, 100 μl of the labeled conjugate solution, either 100 μl or none of the standard thyroxin solution, and a sufficient volume of 0.1 M phosphate buffer (pH 7) to make a total volume of 500 μl. The reaction mixtures were incubated with shaking for two hours at 25°C. Each reaction mixture was then vacuum filtered through a glass wool plugged, dry pasteur pipette previously treated sequentially with periodate and ethylene glycol solutions to eliminate possible FAD contamination. To a 350 μl aliquot of each filtrate was added 117 μl of the monitoring reagent and 50 μl of the apoenzyme solution. After one hour, the absorbance of each reaction mixture was measured at 520 nm.

D. Results

Following is Table 4 showing the results of the assay procedure in measuring thyroxine. The absorbance results are expressed as the average of duplicate runs corrected for residual enzyme activity in the apoenzyme solution (absorbance of 0.467) and for endogenous FAD in the antibody suspension (absorbance of 0.041).

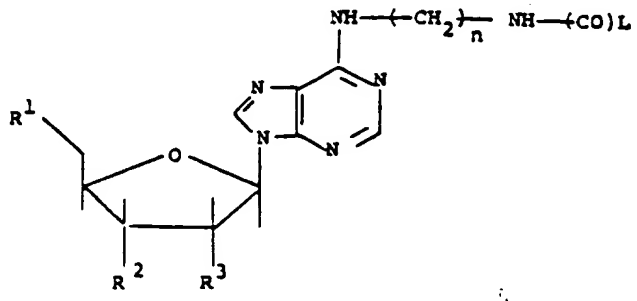
Tabl 4

Volume of Thyroxine Standard Added (μl)	*Absorbance (520 nm)*
0	0.231
100	0.295

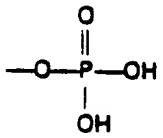
The results demonstrate that the present labeled conjugates are useful in a specific binding assay method for determining a ligand in a liquid medium.

Claims

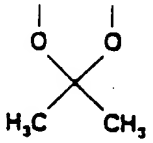
1. A compound of the formula



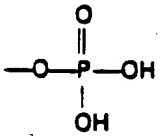
wherein (CO)L is a specifically bindable ligand, or a binding analog thereof, bound through an amide bond; n=2 through 6; R^1 is —OH or



when R^2 and R^3 together form the group



or R^1 is



when R^2 and R^3 are —OH.

2. The compound of Claim 1 wherein said specifically bindable ligand is an antigen or an antibody thereto; a hapten or an antibody thereto; or a hormone, vitamin, or drug, or a receptor or binding substance therefor.

3. The compound of Claim 1 wherein said specifically bindable ligand is an antigenic polypeptide or protein, a hapten, or an antibody.

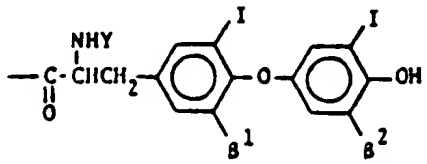
4. The compound of Claim 3 wherein said specifically bindable ligand is an antigenic polypeptide or protein of molecular weight between 1,000 and 4,000,000.

5. The compound of Claim 3 wherein said specifically bindable ligand is a hapten of molecular weight between 100 and 1,500.

6. The compound of Claim 1 wherein said specifically bindable ligand is an iodothyronine hormone.

7. The compound of Claim 6 wherein said hormone is thyroxine.

8. The compound of Claim 1 wherein —(—(CO)L is



wherein Y is an amine-protecting group and β^1 and β^2 are, independently, hydrogen or iodine.

9. The compound of Claim 8 wherein Y is trifluoroacetyl.

10. The compound of Claim 8 or 9 wherein both β^1 and β^2 are iodine.

11. The compound of any of Claims 1 to 10 wherein n=2 or 6.

12. Any one of the compounds of Claim 1 described in the foregoing Examples.

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